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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Prof. Shimon Weiss Dept. of Chemistry and Biochemistry 607 Charles E. Young Dr., Rm. 2037 Los Angeles, CA 90095 / (310)-794-0093	8. PERFORMING ORGANIZATION REPORT NUMBER 4-24946
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14. ABSTRACT

Enhanced peptide-coated qdots (with high brightness & high saturation intensity) were developed. Two high-affinity targeting "velcro-pairs" based on avidin-biotin and fluorescine-antibody interactions were demonstrated and used to specifically target single proteins in membranes of live cells. Single molecule spectroscopy and imaging of individual qdots-labeled lipid rafts receptors were performed. Software tools were developed to analyze individual diffusion and trafficking trajectories. These studies provided strong support to the lipid raft hypothesis. Cloning and fusion of avidin (Avd) to 4 immune synapse components were achieved.
These mutants are being characterized by flow cytometry and fluorescence microscopy.

15. SUBJECT TERMS

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FINAL REPORT

Monitoring Cellular Interactions During T Cell Activation At The Single Molecule Level Using Semiconductor Quantum-Dots

Objectives:

1. Create molecular chimeras with avidin (Avd) insertions for a selective panel of transmembrane proteins that are directly involved in T-Cell Receptor (TCR) signaling.
2. Record and evaluate the movement of qdot tagged molecules into Immune Synapse (IS) using cultured cell lines, and determine the contribution of each components, as well as the cytoskeletal and lipid environment in IS formation.
3. Verify IS profiles in natural populations of murine T cells, and generate IS signature profiles based on the movement of synaptic components in response to different types of stimuli, such as agonist and antagonist.
4. Develop approaches for targeting and delivery of qdots inside living cells.

Status of effort:

Enhanced peptide-coated qdots (with high brightness & high saturation intensity) were developed. Two high-affinity targeting "velcro-pairs" based on avidin-biotin and fluorescine-antibody interactions were demonstrated and used to specifically target single proteins in membranes of live cells. Single molecule spectroscopy and imaging of individual qdots-labeled lipid rafts receptors were performed. Software tools were developed to analyze individual diffusion and trafficking trajectories. These studies provided strong support to the lipid raft hypothesis. Cloning and fusion of avidin (Avd) to 4 immune synapse components were achieved. These mutants are being characterized by flow cytometry and fluorescence microscopy.

Accomplishments/New Findings

Hybrid approach to the synthesis of highly luminescent infrared CdTe/ZnS and CdHgTe/ZnS qdots:

A novel approach for the synthesis of highly luminescent CdTe/ZnS and CdHgTe/ZnS core/shell semiconductor nanocrystals qdots was demonstrated. The approach is based on hybridizing two synthesis routes, leading to novel nanocrystal compositions and small core/shell sizes (4-5 nm) that emit in the far-red and near-infrared regions. These particles exhibit higher resistance to oxidation and photobleaching, have high quantum yields, and could be used for biological labeling and imaging.

Bioactivation and cell targeting of semiconductor CdSe/ZnS qdots with phytochelatin-related peptides:

Synthetic phytochelatin-related peptides were used as an organic coat on the surface of colloidal CdSe/ZnS semiconductor nanocrystals qdots synthesized from hydrophobic coordinating trioctyl phosphine oxide (TOPO) solvents. The peptides were designed to bind to the nanocrystals via a C-terminal adhesive domain. This adhesive domain, composed of multiple repeats of cysteines pairs flanked by hydrophobic 3-cyclohexylalanines, was followed by a flexible hydrophilic linker domain to which various bio-affinity tags could be attached. This surface coating chemistry resulted in small, buffer soluble, monodisperse peptide-coated nanoparticles with high colloidal stability and ensemble photophysical properties similar to those of TOPO coated nanocrystals. Various peptide coatings were used to modulate the nanocrystal surface properties and to bioactivate the nanoparticles. CdSe/ZnS nanocrystals coated with biotinylated peptides efficiently bonded to streptavidin and were specifically targeted to GPI-anchored avidin-CD14 chimeric proteins expressed on the membranes of live HeLa cells.

Development of bright Cd^{+} rich peptide-coated qdots:
The composition and structure of inorganic shells grown over CdSe qdots and rods were optimized to yield enhanced photoluminescence properties after ligand exchange followed by coating with phytochelatin-related peptides. In addition to the peptides imparting superior colloidal properties and providing biofunctionality in a single step reaction, the improved shells and pre-treatment with UV irradiation resulted in high quantum yields for the nanocrystals in water. Moreover, peptide coating caused a noticeable red-shift in the absorption and emission spectra for one of the tested shells, suggesting that exciton-molecular orbital (X-MO) coupling might take place in these hybrid inorganic-organic composite materials.

Comparison of photophysical and colloidal properties of biocompatible qdots using fluorescence correlation spectroscopy:
A number of different surface chemistries have been developed in recent years to render qdots water-soluble and biocompatible. However, most of these surface modifications affect qdots' photophysical properties, calling for a method to simultaneously monitor colloidal and fluorescence properties. Fluorescence correlation spectroscopy (FCS) combined with ensemble spectroscopic methods and Monte Carlo simulations were used to interpret and derive photophysical as well as colloidal properties of four different qdots' surface treatments. Using a novel FCS scheme with alternating laser excitation (ALEX-FCS) at two different intensities, optical gradient forces (optical trapping) were ruled out. Concentration of emitting particles, brightness per particle, saturation intensity, blinking (intermittency), hydrodynamic radius and propensity for aggregation of the different bioconjugated qdots were compared.

Testing the lipid raft hypothesis by single molecule imaging of targeted peptide-coated qdots:
GPI- (lipid-) anchored proteins often associate with sphingolipid-sterol rich microdomains of the cell's plasma membrane called lipid rafts. Rafts play important roles in signal transduction, in the formation of the immune synapse and in T-cell signaling. Understanding the interaction of rafts microdomains with GPI-anchored proteins is of key importance to the fields of membrane biophysics and cell biology. The dynamic interaction of GPI-anchored CD14 receptor fused to avidin (CD14-av) with rafts microdomains in HeLa cells were studied. Wide-field and total-internal-reflection (TIR) single-molecule dynamic imaging experiments were performed to follow individual GPI-anchored CD14-av targeted with peptide-coated qdots. Diffusion of individual CD14-av receptors was tracked over extended period of time in the membrane and during trafficking from the membrane to the Golgi apparatus. Dual color TIR imaging permitted the tracking of single CD14-av receptors in the plasma membrane and the correlation of their diffusion with the distribution of cholera toxin alexa 488 labeled rafts microdomains. The study revealed the existence of 2 equally distributed subpopulation of receptors (1) Slow diffusing/immobile CD14-av receptors associated with raft microdomains and (2) fast diffusing receptors (100 times faster) that avoid rafts microdomains. In some cases, drastic changes of diffusion coefficient for receptors entering and exiting raft microdomains were found. The effects of various cholesterol depletion agents and actin polymerization inhibitors on the different receptors population and their respective localization with raft microdomains were also studied.
Lastly, directed motion of GPI-anchored receptors and rafts microdomains were measured, most likely along cytoskeletal filaments, demonstrating single molecular motor motility assay inside the live cell.

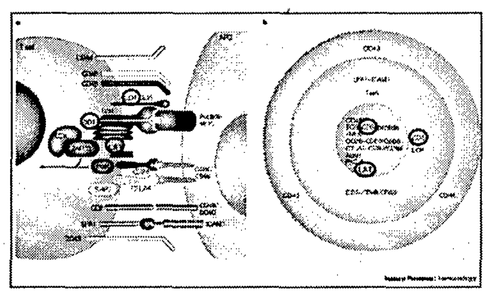
Molecular cloning and fusion of Avidin to immunological synapse (IS) components:
Three well-studied components of the immunological synapse were as candidates for qdot-based imaging: 1) the epsilon chain of the T cell receptor (CD3ε); 2) the CD4 co-receptor; 3) the "Linker for Activation of T cells" (LAT). The mutated form of the CD3ε chain that lacks the immunoreceptor tyrosine-based activation (ITAM) motif was also generated. This truncation reduces the signal transduction potential of the recombinant CD3ε molecule, without affecting its trafficking abilities. A modified form of avidin in which the native (chicken) signal peptide was replaced with a mammalian leader sequence derived from the immunoglobulin kappa light chain (IgK LP) was constructed. This modification has the potential to enhance membrane localization

of the avidin-fusion constructs in mammalian immune cells. To facilitate detection, an epitope tag (HA, derived from the influenza A virus haemagglutinin protein) was inserted at the N-terminus of the recombinant avidin molecule.

The CD3ε, CD4 and LAT constructs were tagged at their N-termini with the IgK LP-HA-Avidin domain using standard molecular cloning technology. The fusions were then cloned into the MSCV retroviral vector, upstream of the IRES-Enhanced Green Fluorescent protein (EGFP) cassette. The IRES element (internal ribosome entry site of the encephalomyocarditis virus) permits both the gene of interest and the EGFP gene to be translated from a single bicistronic mRNA. Retroviruses encoding these constructs were generated and used to transduce the murine T cell hybridoma cell line D011.10. Expression of the fusion proteins was detected by Western Blotting and fluorescent activating cells sorting (FACS) using the HA specific monoclonal antibody. The cellular localization of the constructs was analyzed by confocal microscopy.

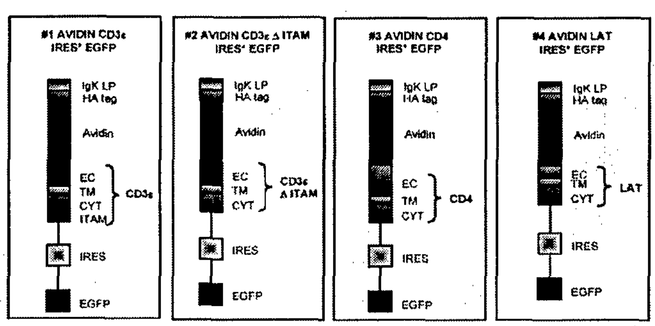
All four constructs were found to localize on the plasma membrane. However, in each case, the fusion proteins formed large aggregates. The topography of these aggregates did not change when we analyzed IS formation in transduced DO11.10 cells incubated with antigen presenting cells (APCs) pulsed with the cognate T cell activating peptide. The natural tendency of avidin to form tetramers together with its known non-specific binding properties could explain the formation of these immobile aggregates.

To solve this problem, non-glycosylated wild-type and monomeric avidin constructs were generated. While these mutations appeared to further alleviate the aggregation problems, the efficiency of fluorescent biotin labeling was low. Furthermore, we observed a clear tendency of some of these constructs to spontaneously internalize following exposure of cells to 37°C, in addition to their failure to localize to the immunological synapse. These results could be explained by the hybrid nature of the DO11.10 system, as well as by the presence in these cells of an endogenous CD4 molecule. Therefore, we are currently extending our studies to a CD4 deficient variant of the Jurkat human T cell leukemia. Alternatively, it is possible that the N-terminal avidin fusion is incompatible with normal trafficking and function of the CD4 molecule. As mention in the progress report, we are continuing our studies to identify other IS components that are more suitable for avidin fusions. We are also exploring the use of non-avidin labeling tags, such as a fluorescein isothiocyanate specific high affinity single chain (sc)Fv specific for fluorescein isothiocyanate.

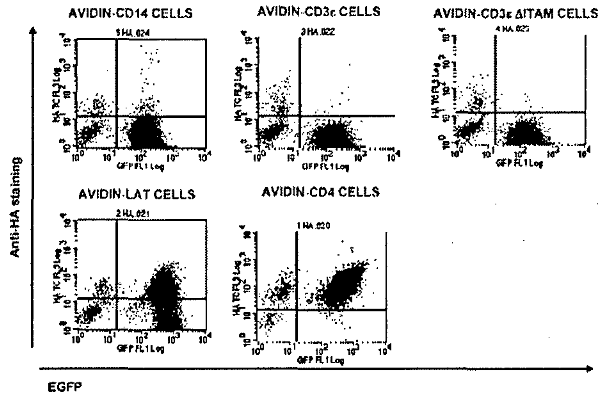


Candidates for avidin-tagging

1. **CD3 components (ε)**
2. **CD4**
3. **Linker for Activation of T cells (LAT)**



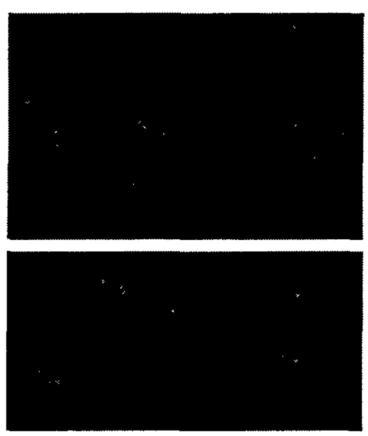
Legend: *IgK LP* - leader sequence of Ig kappa; *HA tag* - influenza A virus haemagglutinin epitope tag; *EC* – extracellular domain; *TM* – transmembrane domain; *CYT* – cytoplasmic domain; *ITAM* - immunoreceptor tyrosine-based activation motif; *IRES* - internal ribosome entry site of the encephalomyocarditis virus (ECMV) permits both the gene of interest and the EGFP gene to be translated from a single bicistronic mRNA.



Internalization of avidin-CD4 labeled with biocytin at 37°C

4°C labeling

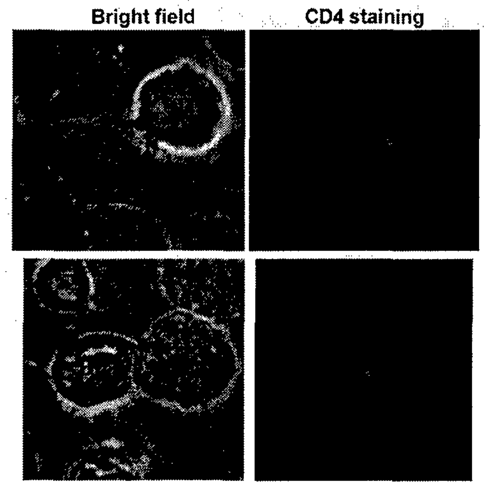
37°C labeling



Avidin tagged CD4 were stably expressed in a murine T cell hybridoma DO11.1O via a retroviral gene overexpression system. For biocytin labeling, cells were washed with phosphate buffered saline (PBS), blocked in 0.1%BSA/PBS for 30min at 4C, then incubated with fluorescent biocytin ( ) for 30 min either at 4C or at 37C. Cells were then washed wiht PBS, fixed by 4% paraformaldehyde and observed using confocal microscopy.

Endogenous CD4 accumulates at the immune synapse

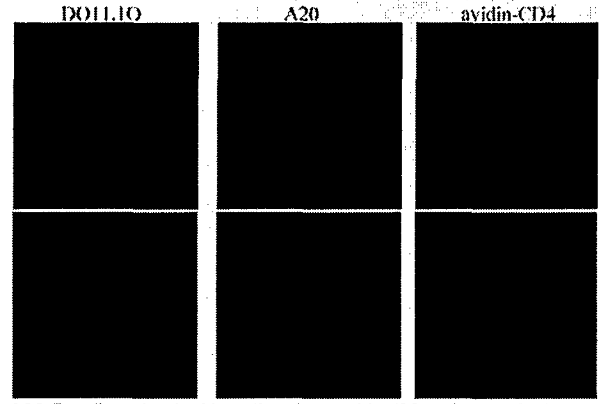
Bright field | CD4 staining



A20 B cells pulsed with antigenic peptide (20 ng/ml, 15 hrs) were mixed with wt DO11.1O cells and incubated at 37C for 30min prior to being fixed. The accumulation of endogenous CD4 at the immune synapse was shown by immunofluorescence staining using antibodies specific for CD4 proteins.

Avidin-CD4 failed to accumulate at the immune synapse

DO11.1O | A20 | avidin-CD4



A20 B cells pulsed with antigenic peptide (20 ng/ml, 15 hrs) were labeled with blue dye CMAC prior to being mixed with DO11.1O cells expressing avidin-CD4 (shown in green). Conjugates were formed after incubation at 37C for 30min. Cells were fixed with 4% paraformaldehyde and avidin-CD4 proteins were labeled using antiobdy specific for avidin (shown in red).

Personnel Supported:

Prof. Shimon Weiss, PI
Prof. Owen Witte, co-PI
Dr. Laurent Bentolila, Associate Researcher 50%
Fabien Pinaud, Graduate Student 50%
James Tsay, Graduate Student 100%
Dr. Caius Radu, Post-doc 100%
Dr. Lili Wang, Post-doc 100%

Publications:

Published:

1. Tsay, J. M.; Pflughoefft, M.; Bentolila, L. A.; Weiss, S., Hybrid approach to the synthesis of highly luminescent CdTe/ZnS and CdHgTe/ZnS nanocrystals. *J Am Chem Soc* **2004,** *126*, (7), 1926-7.
2. Baer, R.; Neuhauser, D.; Weiss, S., Enhanced Absorption Induced by a Metallic Nanoshell. *Nano Letters* **2004,** *4*, (1), 85-88.
3. F. Pinaud, D. King, H.P. Moore, S. Weiss, Bioactivation and Cell Targeting of Semiconductor CdSe/ZnS Nanocrystals with Phytochelatin-related Peptides, *J. Am. Chem. Soc.*; **2004**, *126*, 6115-6123.
4. Michalet, X.; Pinaud, F.; Bentolila, L. A.; Tsay, J.; Doose, S; Li, J.; Sundaresan, G.; Wu, A. M.; Gambhir, S.S.; Weiss, S., Quantum Dots for Live Cells and in vivo Imaging, Diagnostics and Beyond, Science **2005**., 307 (5709), 538-544
5. Tsay, J.; Doose, S.; Pinaud, F.; Weiss, S., Enhancing the photoluminescence of peptide-coated CdSe nanocrystals with shell composition and UV irradiation, J. Phys. Chem B. **2005**, 109, 1669-1674
6. Doose, S.; Tsay, J. M.; Pinaud, F.; Weiss, S., Comparison of Photophysical and Colloidal Properties of Biocompatible Semiconductor Nanocrystals Using Fluorescence Semiconductor Nanocrystals Using Fluorescence Correlation Spectroscopy, Analytical Chemistry, **2005**, 77, 2235-2242

Accepted:

7. Li, J.J.; Tsay, J.; Michalet, X.; Weiss, S., Wave-function engineering: from quantum wells to near-infrared type-II colloidal quantum dots synthesized by layer-by-layer colloidal epitaxy, Chemical Physics (Special Issue on Molecular Nanoscience), accepted, **2005**.

Submitted:

8. Bentolila, L. A.; Weiss, S., Direct Multicolor Fluorescence in situ Hybridization Analysis using Semiconductor Quantum Dot-DNA Conjugates, submitted to Cell Biochemistry and Biophysics

Interactions/Transitions:

a. Participation/presentations at meetings, conferences, seminars, etc.

ARIZONA STATE UNIVERSITY	Single Molecule Nanoscale Rulers
LAWRENCE BERKELEY NATIONAL LAB, SECOND MOLECULAR FOUNDRY USER WORKSHOP	Single Molecule Nanoscale Rulers
CBST MEETING, SACRAMENTO	Single Molecule Nanoscale Rulers
GTL WORKSHOP, WASHINGTON, D.C.	Single Molecule Nanoscale Rulers
DARPA WORKSHOP, ARLINGTON, V.A.	Single Molecule Nanoscale Rulers
HHMI-MAX PLANCK SOCIETY MEETING,	In-vitro and in-vivo single molecule imaging and spectroscopy

MUNICH	
ACS NATIONAL MEETING, PHILADELPHIA	In-vitro and in-vivo single molecule imaging and spectroscopy
SHEWANELLA FEDERATION MEETING WOOD'S HOLE, M.A.	In-vitro and in-vivo single molecule imaging and spectroscopy
OREGON HEALTH AND SCIENCES UNIVERSITY	In-vitro and in-vivo single molecule imaging and spectroscopy
DARPA MEETING, VAIL, C.O.	In-vitro and in-vivo single molecule imaging and spectroscopy
YALE UNIVERSITY, JOSEPH E. COLEMAN MEMORIAL LECTURE	In-vitro and in-vivo single molecule imaging and spectroscopy
POMONA COLLEGE	In-vitro and in-vivo single molecule imaging and spectroscopy
LEICA ADVANCED MICROSCOPY COURSE BOSTON	In-vitro and in-vivo single molecule imaging and spectroscopy
LLNL FRONTIERS IN CHEMISTRY	In-vitro and in-vivo single molecule imaging and spectroscopy
UCSD PHARMACOLOGY SEMINAR	In-vitro and in-vivo single molecule imaging and spectroscopy
UCLA MBI SEMINAR	In-vitro and in-vivo single molecule imaging and spectroscopy
UCLA PHARMACOLOGY SEMINAR	In-vitro and in-vivo single molecule imaging and spectroscopy

b. Consultative and advisory functions to other laboratories and agencies, especially Air Force and other DoD laboratories. Provide factual information about the subject matter, institutions, locations, dates, and name(s) of principal individuals involved.

2002-to date - Lawrence Berkeley National Laboratory Molecular Foundry Advisory Board

2002- to date - Program Advisory Committee of the National Science Foundation Center for "Biophotonics Science and Technolgy"

c. Transitions. Describe cases where knowledge resulting from your effort is used, or will be used, in a technology application.

A start-up company was established and a IP licensing agreement with UCLA was signed.

New discoveries, inventions, or patent disclosures:

(under NIH grant)

1) "Bioactivation of particles", F. Pinaud, D. King and S. Weiss, US provisional 60/378,720.
2) "Synthesis of near-IR emitting core-shell nanocrystals for in vivo biological applications", J. Tsay, M. Pflughoefft and S. Weiss, US provisional 60/505,461.
3) "Hybrid approach to the synthesis of highly luminescent CdTe/ZnS and CdHgTe/ZnS", J. Tsay, M. Pflughoefft and S. Weiss, US provisional 60/538,049.

4) "Bioactivation and cell targeting of semiconductor CdSe/ZnS nanocrystals with phytochelating-related peptides", F. Pinaud, D. King and S. Weiss, US provisional 60/558,511.
5) "Bioactivation continuation (+Cd)", Tsay et al, US provisional xxx.

Honors/Awards:

The 2005 The Rank Prize in opto-electronics
The 2001 Michael and Kate Barany Biophysical Society Award
Fellow of the Optical Society of America (1999).